REMARKS

The background of the invention, brief summary of the invention, brief description of the drawings, and abstract of the disclosure sections of the specification submitted on 11/23/2004 were kept, as there were no, or only minor corrections to be made to these. A substitute (new) detailed description of the invention section was submitted here, as a correction of the old was too marked up. References to the location of text to support the claims, occasionally refer to the existing specification (background of the invention, brief summary of the invention; and therefore existing specification page numbers), but mostly to the newly submitted detailed description of the invention (using page numbers in this amendment).

Amended drawings were submitted with the 11/23/2004 amendment of this application. Also, one of the original drawings was canceled. In the office action of 10/12/2006, the examiner makes no mention as to whether or not the amended drawings are approved. However, a telephone conversation with the examiner on 12/08/2006 indicated that they were approved. As such, alterations in the currently submitted detailed description of the invention, from the original, were made to adapt the text describing the drawings to the amended versions of those drawings.

Specific Comments to the Examiner's Objections:

Objection 5 a):

The examiner objects to the use of the term fluorescein or fluorescein-conjugated in describing the hapten-ligands used in the brief description of the drawings section. These were previously described using the acronym FITC. Since the meaning of FITC is: flourescein isothiocyanate, and when FITC is conjugated to a protein it is commonly referred to as FITC-protein, or FITC-conjugated protein, or fluorescein-protein, or fluorescein-conjugate-protein, it was thought that the use of fluorescein here was merely synonymous with FITC. This is widely known in the art, however, the applicant did not realize the non-obvious nature of the definition.

To correct this, these references to fluorescein-proteins and fluorescein-conjugated proteins have been deleted and replaced with the originally used FITC-protein or FITC-conjugated protein terms.

Objection 5 b):

The examiner objects to the detailed description of the invention section in the substitute specification submitted 11/23/2004, as it contains new matter in various areas. These were not previously recited in the originally submitted specification.

To correct this, the detailed description submitted 11/23/2004 has been deleted and replaced with the original. With the original submission, the detailed description of the drawings was improperly placed in the brief description of the drawings section. These entries have been moved verbatim to the original detailed description of the invention

section, so that all detailed descriptions of the method are together – but yet still original. Other minor corrections to the original detailed description of the invention were made. A list of these corrections and a detailed list of the contents of the current detailed description of the invention, and their origin in the originally submitted application, follows at the end of the remarks.

Objection 5 c):

The examiner objects to the addition of a sentence added to the abstract whereby the description of the use of standards was mentioned, as this was deemed to add new matter. The discussion of standards and their use was made throughout the original specification. It was thought the addition of this to the abstract helped to teach the method.

To correct this, the added sentence has been struck out.

Objection 5 d):

The examiner objects to a paragraph added to the detailed method of the invention, wherein the substitute reagents are mentioned.

To correct this, in the newly submitted detailed method of the invention, this paragraph has been deleted.

Claims Objections

Objection 7:

The examiner objects to the method claim as to not being supported by the specification. The specification does not convey that the inventor had possession of the invention as stated in the claims. The claims contain terms and methods not stated in the original submission. The inventor attempts to support claims by the specification filed 11/23/2004, however this specification is objected to, so the support is moot. The identity and/or structural characteristics of ligands, reagents, and method steps are not pointed out.

To correct this, new claims have been filed. The new claims parallel the old to a high degree. The major method claim (97) has been narrowed considerably, so that it only utilizes reagents and steps spelled out in the original specification. All terms used in all claims, and their origins in the new and original specification are cited below.

New language to make use of standards and the standard curve are entered so as to teach the invention. The nature of the signal has been limited to only that mentioned in the specification. The organization of the method and the application steps is more clearly laid out. The term unknowns has been dropped. The goal of the preamble is now elucidated.

The precise use of and the definitions of all reagents, incubation steps, voltages, membranes, membrane dimensions, film exposure, etc. are stated in detail in the specification, for the measurement of FITC-Tf binding to cells: page 22, line 9 to page

25, line 20 (of this amendment). Thus the new method claim 97 in conjunction with these originally submitted procedures point out the identity and/or structural characteristics of ligands, reagents, and method steps. The ligands used here are complex proteins and glyco-proteins of 6,000 – 80,000 in molecular weight. A defined structure for these is extremely difficult to represent.

Objection 8. a.)

The preamble of the method claim 75 was not achieved by the method. There is a lack of correlation between evaluating hapten-ligand bound in the preamble; to determining the amount of hapten-ligand bound as the final method.

This has been corrected by a re-stating of the method claim (now claim 97). The claim's preamble is now: "method for a quantification of ligand binding to a surface". The final recited step is: "whereby the quantifying of the amount of the membrane-bound hapten-ligand arising from said lysate, is used to quantify the amount of said hapten-ligand originally bound to said surface." Therefore, the goal of quantification is met by the final step of quantifying. The statement of the method here closely reflects that stated in the abstract: "a procedure for measuring the binding of an entity (ligand) to a surface."

Objection 8. b.)

The method claim 75 is unclear as to what the term "signal" refers to.

This has been corrected in the new method claim 97, which is now: "the applying of

a color or light-producing substrate for said enzyme, (is performed,) thereby producing a signal." Thus, the signal is now stated as color or light, the two signals mentioned in the specification, and widely known in the art to be produced by such enzymes.

Objection 8. c.)

The method claim 75 in unclear as to what the term "known quantity refers" to.

This term was unnecessary to convey the method, therefore, it was deleted.

Objection 8. d.)

Claims 79, 86, 89, and 92 use improper Markush group format. The phrase: "wherein said X is selected from the group consisting essentially of A, B, C, and D", is objected to.

To correct this, all cases of this have been changed to: wherein said X is selected from the group consisting of A, B, C, and D.

Objection 9.

The publication of Cavanaugh et al., (1998) is a printed publication wherein the invention is described and taught more than 1 year prior to the submission of this application. Note: Based on the page numbers cited by the examiner, it presumed that the

publication cited is Cavanaugh and Nicolson (1998).

There are multiple reasons why Cavanaugh and Nicolson (1998) does not teach the method of this application. The applicant draws attention to the fact that the claims now require the use of standards in the method. The strength of the invention is the ability of users to calculate the amount of hapten-ligand in lysate samples on the membrane, by comparison to signals obtained from known amounts of the hapten-ligand standards applied to the membrane. Both methods in the prior art example are not, and could not be standardized.

The first method cited in Cavanaugh and Nicolson is that of immunofluorescent staining of cell surface transferrin receptor using a phycoerythrin-labeled antibody to the transferrin receptor. Here, the anti-transferrin receptor antibody acts a ligand for the transferrin receptor, and the cells act as the binding surface, which contain said receptor.

However, in Cavanaugh and Nicolson the bound phycoerythrin-labeled antibody is detected by flow cytometry. To match the invention, Cavanaugh and Nicolson would have had to solubilize, or make a lysate of the phycoerythrin-labeled anti-transferrin receptor antibody-cell combination; and apply to a membrane, the lysate in conjunction with standard quantities of the ligand, and detect the membrane-bound antibody using an enzyme conjugated anti-phycoerythrin. Then, they could have used the signals from the standard quantities of phycoerythrin-labeled anti-transferrin receptor antibody to

construct a standard curve, so as to accurately quantify the amount of antibody originally bound to the cells. As presented, Cavanaugh and Nicolson use flow cytometry and can only display relative differences in ligand binding by the different cells. This cited method **does not** use applying to a membrane, **does not** use hapten-ligand standards, and **does not** use an enzyme signal (it uses fluorescence), therefore, it does not teach the method in this application.

The second method cited in the prior art of Cavanaugh and Nicolson which approximates the invention, is that of affinity isolation of biotinylated transferrin receptor. Here, the labeled ligand is not a preparation of a pure labeled ligand, but a complicated mixture of the entire population of all biotinylated cell surface constituents. This is applied to an immobilized receptor: transferrin-agarose (the surface) which will hopefully recognize only one of the biotinylated entities in the mixture (the biotinylated -transferrin receptor = the hapten-ligand). The ligand mixture is applied to the surface, ligand-surface complex is washed, bound components released (solubilized), separated by electrophoresis, blotted, and detected using an enzyme conjugated avidin/streptavidin. A major band at the expected size of the preferred ligand was seen and presumed to be that only that transferrin receptor ligand. However, there was no guarantee of that. Other biotinylated cell surface components of similar molecular weight, which bound to the receptor (surface) would also be present in this band. In addition, because the labeled ligand exists in a potpourri of other biotinylated entities, and no pure ligand exists, it is impossible to standardize the system and calculate the amount of putative ligand bound to the receptor. Again as presented, Cavanaugh and Nicolson can only display relative

differences in biotinylated transferrin receptor content in the biotinylated cell surface solutions from the different cells. With this cited assay, the reagents are reversed from the one that the patent describes. In the citation, the ligand preparation is a mixture of unknown labeled entities, and the insoluble surface a characterized population of single receptors. In the preferred embodiment of this patent application, the ligand exists as one defined pure entity, and the insoluble surface to which it binds is an unknown of multiple receptors, one or more of which binds the pure ligand. In addition, the method in this application requires that the membrane-bound hapten-ligand be detected by the application of an antibody to the hapten. The method of Cavanaugh and Nicolson (1998) utilizes avidin or sreptavidin to detect the biotin label on the ligand. Avidin and streptavidin are not antibodies.

Thus, the second method of Cavanaugh and Nicolson (1998) **does not** teach the method of this application because: 1.) it **does not** use hapten-ligand standards, 2.) it **does not** use a standard curve, 3.) it **does not** and **can not** quantify the weight amount of hapten-ligand bound to the surface, and 4.) it **does not** use an antibody to the hapten.

Citations of Claims Terms In the Specification:

Claim 97: Terms: ligand, surface

Page 10, line 4 original submission:

Page 7, line 3 current specification (brief summary of invention section):

The present invention relates to the need in biological research to measure the ability of cells or other **surfaces** to bind a given compound (hereafter referred to as a **ligand**). The **ligand** could be a growth factor or any other factor whose study involves the need for persons to assess the ability of cells, or any other insoluble particle or material, to bind it.

Claim 97: Term: hapten-conjugated ligands

Page 10, line 11 original submission:

Page 7, line 10 current specification (brief summary of invention section):

The invention makes use of many available anti-hapten antibodies which specifically recognize a **hapten-conjugated binding entity** or **ligand (hapten-ligand)**

Claim 97: Term: lysate

Page 20, line 6 of the original submission:

Page 23, line 2 of the current detailed description submission:

Cells were incubated with the lysing solution for 30 min at 4°C and all **lysates** were

pipetted into separate 1.5 ml conical tubes.

Claim 97: Term: membrane

Page 24, line 8 of the original submission:

Page 32, line 20 of the current detailed description submission:

Bound hapten-ligand is then solubilized (removed) and applied to a **membrane** support or separated by electrophoresis and applied to a **membrane** support.

Also:

Page 20, line 15 of the original submission:

Page 23, line 11 of the current detailed description submission:

A 14 X 14 cm nitrocellulose **membrane** was equilibrated in transfer buffer and the gel and **membrane** assembled into a transfer apparatus and immersed in transfer buffer. Gel components were transferred to the **membrane** at a constant voltage of 40 V for 1.5h.

Claim 97: Term: standards

Page 16, line 3 of the original submission:

Page 29, line 2 of the current current detailed description submission:

For the **standards,** increasing volumes (2, 4, 8, and 16 μL) of a 100 ηg/ml FITC-Con A solution were applied to a nitrocellulose membrane.

Also:

Page 12 line 22 of the original submission:

Page 25 line 15 of the current detailed description submission:

Figure 2C shows results from a blot treated as in Figure 2A but loaded with pure FITC-TF **standards** in the amounts (in ηg) indicated on the top

Also:

Page 24 line 19 of the original submission:

Page 33 line 8 of the current detailed description submission:

The procedure allows for easy **standardization** as different user-definable levels of a **standard** solution of the Hapten-ligand can be simultaneously applied to the electrophoresis gel or to the dot-blot or slot-blot membrane.

Claim 97: Term: membrane-bound hapten-ligand

Page 24, line 8 of the original submission:

Page 32, line 20 of the current detailed description submission:

Bound hapten-ligand is then solubilized (removed) and applied to a membrane support or separated by electrophoresis and applied to a membrane support. The **membrane-bound hapten-ligand** is detected

Claim 97: Term: enzyme-conjugated antibody

Page 24, line 10 of the original submission:

Page 32, line 21 of the current detailed description submission:

The membrane-bound hapten-ligand is detected by application of an **enzyme-conjugated antibody** to the hapten;

Claim 97: Term: color or light-producing substrate

Page 24 line 13 of the original submission:

Page 33 line 2 of the current detailed description submission:

The resultant membrane-associated enzyme is detected and quantified by the application of a **color or light-producing substrate** which reacts with the enzyme.

Claim 97: Terms: signal, standard curve

Page 21, line 16 of the original submission:

Page 24, line 11 of the current detailed description submission:

The **signal** returned from the imager is plotted against the amount of FITC-Tf contained in the band and a **standard curve** is constructed to calculate the amounts of FITC-Tf bound by the cells.

Claim 97: Term: signal

Page 24, line 18 of the original submission:

Page 33, line 7 of the current detailed description submission:

dual antibody incubation steps amplify the **signal** significantly.

Claim 98: Term: un-conjugated ligand: this is not stated per se, however it is used in the description of specific ligands:

Page 15, line 14 of the original submission:

Page 28, line 13 of the current detailed description submission:

One well of each FITC-Con A concentration also received 200 μg/ml of native (**un-conjugated** Con A)

Also:

Page 18, line 3 of the original submission:

Page 31, line 3 of the current detailed description submission:

this is reduced significantly when excess **un-conjugated** insulin was present.

Claim 99: Term: electrophoretically separated lysate

Page 14, line 6 of the original submission:

Page 28, line 15 of the current detailed description submission:

The lysates were centrifuged at 5,000 X g for 5 min., and the supernatants were separated by SDS-PAGE.

Claim 99: Term: electrophoretically separated standards

Page 13, line 1 of the original submission:

Page 25, line 17 of the current detailed description submission:

The pure FITC-Tf samples were electrophoresed,

Claim 100: Term: SDS-PAGE

Page 15, line 14 of the original submission:

Page 23, line 8 of the current detailed description submission:

Treated samples were loaded onto a 12 X 12 cm 10% acrylamide **SDS-PAGE** electrophoresis gel

Also:

Page 14, line 7 of the original submission:

Page 27, line 19 of the current detailed description submission:

The lysates were centrifuged at 5,000 X g for 5 min., and the supernatants were separated by **SDS-PAGE.**

Claim 100: Term: electrophoresis according to Schagger Von Jagow

Page 17, line 19 of the original submission:

Page 30, line 17 of the current detailed description submission:

were separated by SDS-**PAGE run according to Schagger-Von Jagow**

Claim 100: Term: agarose electrophoresis

Page 23, line 13 of the original submission:

Page 31, line 11 of the current detailed description submission:

After binding, the bound PCR product is released through heat de-naturation, is separated by **agarose electrophoresis,**

Claim 101: Terms: antibody to the hapten, enzyme-conjugated antibody to the anti-hapten antibody

Page 24, line 11 of the original submission:

Page 33, line 1 of the current detailed description submission:

or by application of an **antibody to the hapten** followed by application of an **enzyme-conjugated antibody to the anti-hapten antibody**.

Claim 102: Term: biological cells. This is not defined per se, however, it is a term widely known in the art. references to this is made numerous times as "cells". An allegorical reference to this is:

Page 10, line 1 of the original submission:

Page 7, line 3 of the current specification (brief summary of the invention section):

The present invention relates to the need in **biological** research to measure the ability of **cells** or other surfaces to bind a given compound

Also:

Page 18, line 6 of the original submission:

Page 22, line 5 of the current detailed description submission:

to **cell** surfaces

Claim 104: Terms: biotin, rhodamine, and digoxygenin

Page 22 line 1 of the original submission:

Page 32 line 2 of the current detailed description submission:

In particular, anti-**digoxygenin**, anti-**rhodamine** and anti-**biotin** antibodies exist which would recognize ligands conjugated with **those compounds**.

Claim 104: Term: fluorescein

Page 19, line 6 of the original submission:

Page 22, line 5 of the current detailed description submission:

The basic detailed method using **fluorescein**-conjugated transferrin

Claim 105, 106: Terms: protein, biological factor

The term "biological factor" is not defined per se, however, the use of the word "factor" as an unknown entity is widely used in the life sciences art. This is referred to allegorically:

Page 1, line 20 of the original submission:

Page 2, line 2 of the current specification (background of the invention section):

Frequently, researchers desire to analyze the ability of various **proteins** and other **factors** to bind to cell surfaces. Usually, the type of binding studied is one where the binding **factor**

Claim 107: Terms: transferrin, concanavalin A, avidin, annexin V, and insulin

Page 19, line 4 of the original submission:

Page 22, line 2 of the current detailed description submission:

Specifically, the method developed measures the binding of **transferrin, concanavalin-A, avidin, annexin-V, and insulin** to cell surfaces.

Claim 108: Term: DNA

Page 23, line 8 of the original submission:

Page 31, line 8 of the current detailed description submission:

The assay could be used to verify the hybridization of biotin-labeled **DNA** to other DNA molecules.

Claim 109: Term: blotting

Page 31, line 13 of the original submission:

Page 34, line 19 of this amendment (abstract of the disclosure):

A combination of the use of anti-hapten antibodies along with membrane-**blotting** technologies to assess hapten-ligand binding to surfaces

Claim 110: Term: electro-blotting

Page 13, line 12 of the original submission:

Page 26, line 21 of the current detailed description submission:

In Figure 3G and Figure 3H, both samples are electrophoresed and the gels **electro-blotted** (Figure 3I).

Also:

Page 23, line 13 of the original submission:

Page 31, line 12 of the current detailed description submission:

electro-blotted to nytran,

Claim 110: Term: dot blotting

Page 16, line 2 of the original submission:

Page 29, line 1 of the current detailed description submission:

Figure 6 shows results obtained when the samples from Figure 5 were analyzed by a **dot blot** procedure

Claim 110: Term: slot blotting

Page 24, line 22 of the original submission:

Page 33, line 10 of the current detailed description submission:

simultaneously applied to the electrophoresis gel or to the dot-blot or **slot-blot** membrane.

Claim 110: Term: Western blotting

Page 4, line 4 of the original submission:

Page 4, line 7 of the current specification (background of the invention):

Western blotting is a technique where cell lysates obtained by detergent treatment are separated by electrophoresis and the separated components contained within the electrophoresis gel are driven onto a protein-binding membrane via electric current.

Claim 111: Term: conventional transfer membrane

Page 22, line 8 of the original submission:

Page 32, line 9 of the current detailed description submission:

It must also bind to a **conventional transfer membrane**

Claim 112: Term: DNA binding membranes

DNA binding membranes are not defined per se. However, a method for DNA is described. As DNA must be bound to a membrane to perform the method, any DNA binding membrane would suffice.

Claim 112: Term: protein binding membranes

Page 4 line 6 of the original submission:

Page 4 line 9 of the current specification (background of the invention):

separated components contained within the electrophoresis gel are driven onto a **protein-binding membrane** via electric current.

Claim 113: Term: nitrocellulose

Page 20 line 15 of the original submission:

Page 23 line 11 of the current detailed description submission:

A 14 X 14 cm **nitrocellulose** membrane was equilibrated in transfer buffer and the gel and membrane assembled into a transfer apparatus and immersed in transfer buffer. Gel components were transferred to the membrane at a constant voltage of 40 V for 1.5h.

Claim 113: Term: nytran

Page 23, line 13 of the original submission:

Page 31, line 13 of the current detailed description submission:

electro-blotted to **nytran**, and is detected by incubation

Claim 114: Term: horseradish peroxidase

Page 6, line 4 of the original submission:

Page 23, line 20 of the current detailed description submission:

horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG

Claim 115: Term: photographic film

Page 21, line 6 of the original submission:

Page 24, line 2 of the current detailed description submission:

loaded into an X-ray film cassette along with an 8 X 10 inch piece of **photographic film.**

(Trademark product Amersham ECL hyperfilm substituted with photographic film for this submission.)

Claim 115: Term: Imager

Page 21 line 9 of the original submission:

Page 24 line 4 of the current detailed description submission:

Bands produced on the film were quantified using an **imager**.

(Trademark product Bio-Rad Multi Imager substituted with imager for this submission.)

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Changes to the Detailed Description of the Invention:

1.) The descriptions for figures 1, 2, and 3 have changed, as these figures were amended. These have changed only slightly, to reflect the amended figures, as the new 10 figures contain new sections.

2.) Insertions of text into the original detailed description of the invention, consist of descriptions of the drawings. These blocks of text were present in the originally submitted application, but they were incorrectly placed in the brief description of the drawings section. These have changed only slightly here, to reflect the amended figures. 15 The sites of these insertions (using page and line numbers from this amendment) into the currently submitted detailed description of the invention, and their locations in the original specification are:

2a.) Description for figure 1: page 24, line 15 to page 25, line 4. In the original 20 specification this was on: Page 12, line 3 to 11.

2b.) Description for figure 2: page 25, lines 6 to 20. In the original specification this was on: page 12, line 13 to page 13, line 4.

2c.) Description for figure 3: page 26, line 15 – page 27, line 9. In the original specification this was on: Page 13, line 6 to 19.

2d.) Description for figure 4: page 27, line 11 - page 28, line 5. In the original specification this was on: page 13, line 21 to page 14, line 14.

2e.) Description for figure 5: page 28, line 7 to 22. In the original specification this was on: Page 15, line 7 to line 22 (description for original figure 6 - the original figure 5 was canceled, so subsequent new figure numbers are decreased by 1:).

2f.) Description for figure 6: page 29, lines 1 to 11. In the original specification this was on: page 16, line 2 to line 11 (description for original figure 7).

2g.) Description for figure 7: page 29, line 13 to page 30 line 5. In the original specification this was on: page 16, line 13 to page 17, line 5 (description for original figure 8).

2h.) Description for figure 8: page 30, line 7 to page 31, line 6. In the original specification this was on: Page 17, line 7 to page 18, line 5 (description for original figure 9)

Other minor alterations of the original detailed description of the invention section were made to correct typographical and other errors. These are outlined here:

3.) In various places, the symbols for nano (η) and micro (μ), were denoted by n and u, respectively. These have been corrected.

4.) The original text on page 19, line 8:

"Specific alterations of this procedure for other ligands are described in the description of figures section."

Was changed on page 22, line 6 of the currently submitted detailed description to:

"Specific alterations of this procedure for other ligands follow."

5.) On page 22, line 9, (FITC-Tf) was added so as to define that acronym as fluorescein-conjugated iron-saturated (holo) human transferrin, as soon as possible. FITC-Tf is used on line 18 without prior definition in the method.

This is defined on page 5, line 8 of the original submission: "fluorescein-labeled (or FITC-labeled) transferrin (hereafter referred to as FITC-Tf)".

6.) Amersham as the source of ECL HRP substrate was deleted, as it is a trademark name. ECL HRP substrate is well known in the art and is widely available from a number of sources. Any of these will suffice for the method. Likewise, reference to Bio-Rad as the source of the multi imager used was dropped. A number of instruments for measuring the light produced from ECL substrates on membranes are available, and are widely known in the art. Any of these will suffice for the method. The term hyperfilm was also replaced with the generic term: photographic film. Hyperfilm is a specific trademarked product produced by Amersham. A number of films for imaging the light produced from ECL substrates are available and are widely known in the art. Any of these will suffice for the method. The term Coulter has been deleted. This is a trademark name used for certain cell counters. A number of cell counters are known in the art and are widely available. Any of these will suffice for the method. These instruments and reagents were used for the invention only because they were on hand. There is nothing specific about them that dictate only their use. The Bio-Rad multi-fluor S imager used is now out of production.

7.) The acronyms HRP (horseradish peroxidase) and ECL (enhanced chemiluminescence) are widely known in the art. In the newly submitted detailed description, HRP is re-defined as horseradish peroxidase on page 23, line 20, and ECL is re-defined as enhanced chemiluminescence on page 24, line 1, so as to define these acronyms and make the method clear:

"horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG for 2h at 25°C and washed again. Each membrane was covered with an enhanced chemiluminescence (ECL)"

The term enhanced chemiluminescence was present on page 11 line 1 of the original submission:

For maximum sensitivity, a light-producing substrate is applied and the enzyme is detected by **enhanced chemiluminescence** (ECL).

The term horse radish peroxidase was defined on page 6 line 4 of the original submission:

The membrane was washed and incubated with goat **horse radish peroxidase** (HRP)-conjugated anti-rabbit IgG

8.) In the original submission, on Page 19 line 21, the text:

from 0.02 to 0.5 μg/ml final FITC-Tf

was changed,

In the current submission, now on Page 22 line 18 to:

from 0.06 to 2.0 μg/ml final FITC-Tf.

The range of concentrations used was changed to match the amended figure 2.

9.) In the original submission, on page 17, line 4, the text:

specific Con A bound

was changed in the current submission, now on page 30, line 4 to:

specific Avidin bound

Con A was a typographical error here as this is in the Avidin section.

10.) Throughout, the improper term quantitated has been replaced by quantified. Also quantitate has been replaced by quantify.

11.) General comments were moved to the end of the detailed description, after all figure descriptions. These consist of:

11a.) Discussion of DNA techniques: page 31, line 8 to line 17. In the original specification this was on: page 23, line 8 to line 18.

11b.) Discussion of dot blot techniques: page 31, line 19 to line 22. In the original specification this was on: page 23, line 20 to page 24, line 2.

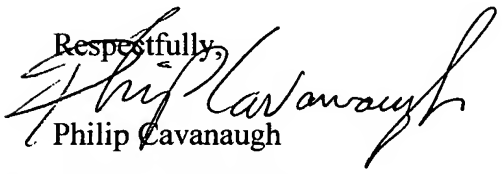
11c.) Discussion of general applications: page 32, line 1 to 14. In the original specification this was on: page 21, line 21 to page 22, line 13.

Conclusion

The applicant now believes that the specification and claims are in proper form, and that all of the examiner's objections have been satisfactorily answered, that the prior art challenge has been answered.

The applicant wishes to thank the examiner for the reviews to date and hopes that this amendment elucidates the method, is easy to follow, and can result in some allowance.

Respectfully,



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In the Specification (*continued*):

Please delete the previous detailed description of the invention (page 11, line 1 to page 26, line 14 - of the previously submitted specification). This is shown on the following pages as struck out, to precisely indicate the deleted text:

~~**DETAILED DESCRIPTION OF THE INVENTION:**~~

~~The object of the present invention is to provide a method for the sensitive non-radioactive assessment of ligand binding to insoluble surfaces. Specifically, the method developed measures the binding of transferrin, concanavalin A, avidin, annexin V, and insulin to cell surfaces.~~

~~A schematic of the detection and competitive binding strategy of the assay is shown in Figure 1. In Figure1A, a cell monolayer is exposed to a solution of fluorescein labeled transferrin (FITC-Tf). In Figure 1B, an identical cell monolayer is exposed to a solution of FITC-Tf plus an excess of unlabeled transferrin. In either case, 3 molecules of Tf bind per cell. When washed (Figure 1C) and lysed, cells from Figure 1A produced a lysate containing 9 molecules of FITC-TF (Figure 1E), whereas cells from Figure 1B produce a lysate containing 1 molecule of FITC-TF (Figure 1D, Figure 1F). In Figure 1G, both samples are loaded onto an electrophoresis gel, along with standards containing increasing levels of known amounts of FITC-Tf. When electrophoresed, the Tf and cell lysate proteins stack up and migrate according to their molecular weight (Figure 1H). These are blotted onto a membrane as shown in Figure 1I, where their relative positions are maintained. The membrane is blocked and treated with goat anti-FITC (Figure 1J), which specifically binds to the FITC-Tf only. The membrane is washed and treated with anti-goat IgG-peroxidase, which recognizes only the goat anti-FITC (Figure 1K). The membrane is washed again and treated with an enhanced chemiluminescent (ECL) substrate for peroxidase (Figure 1L), where light produced per band correlates with~~

~~membrane FITC-Tf content per band. The light produced is recorded on an X-ray film (Figure 1M), which is imaged so that each band is assessed for optical density/mm^2 (ODu/mm^2; Figure 1N). With the cells from Figure1A, a large band is seen on the X-ray film exposed by this light, at the same vertical position as the standard signals. Thus, the detection of the ligand is seen, at the correct molecular weight for transferrin. The amount of FITC-Tf in this band can be estimated by comparing its signal to that of the standards. The amount of FITC-Tf bound per cell is then calculated, from the cell density of the culture plate used in Figure 1A. When the FITC-Tf band produced from the cells from Figure1B is analyzed, minimal light production is seen. Thus, competition for Tf binding to the cells between FITC-Tf and Tf is seen, demonstrating specific binding to the cells by the FITC-Tf.~~

~~The basic detailed method using fluorescein conjugated transferrin as a detectable antibody recognizable hapten tracer follows:~~

~~Fluorescein conjugated iron saturated (holo) human transferrin was obtained from commercial sources. Cultured cells to be measured were grown to 50 – 60 % confluence in 12 well plates. Cells were incubated with serum free minimal essential media (alpha modification; ∝-MEM) for 12 h and then again with fresh ∝-MEM for another 12 h. The cell number in three wells was determined by trypsinization of those cells followed by enumeration on a cell counter. Media in remaining wells was replaced with 1 ml binding buffer (BB) which consisted of: 25 mM HEPES in ∝-MEM containing 2mg/ml of bovine serum albumin (BSA); pH 7.5. The cell wells were then allowed to equilibrate to 4°C in a refrigerator. Sets of 5 replicate wells received increasing amounts of FITC-Tf, from~~

~~0.06 to 2.0 µg/ml final FITC-Tf. Two wells of each FITC-Tf concentration set then received unlabeled holo-human transferrin so that the final unlabeled Tf concentration was 100 µg/ml. After a 2h incubation at 4°C, all media was saved (= unbound samples), and the wells were all washed 4 times by the addition and drainage of 1 ml of 4°C phosphate-buffered saline (PBS). All wells then received 0.5 ml of an RIPA cell lysing solution which consisted of PBS containing 1% v/v nonidet P-40 detergent, 0.5% v/v deoxycholic acid, 0.1% v/v sodium dodecyl sulfate (SDS), 100 µg/ml phenylmethyl sulfonyl chloride, and 0.1 units/ml Aprotinin. Cells were incubated with the lysing solution for 30 min at 4°C and all lysates were pipetted into separate 1.5 ml conical tubes. The tubes were centrifuged at 5,000 X g for 10 min and 400 µL of each supernatant was transferred to a fresh tube. All of these tubes received 166 µL of a 4X concentrate sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) treatment solution (0.5 M Tris, 8% w/v SDS, 2% v/v beta-mercaptoethanol, 1.0% w/v bromophenol blue, 20% v/v glycerol, pH 6.8), and were treated at 95°C for 10 minutes. Treated samples (150 uL each) were loaded onto an acrylamide SDS-PAGE electrophoresis gel. The gels consisted of a 12 X 10 cm separating gel containing 0.375 M Tris, 0.1% w/v SDS, 10 % w/v acrylamide, pH 8.8; and a 12 X 2 cm stacking gel containing 0.125 M Tris, 0.1% w/v SDS, 4% w/v acrylamide, pH 6.8.~~

~~Unbound samples were treated similarly to cell lysate samples, and loaded onto said electrophoresis gel. Typically, these have to be diluted 1:10 – 1:100 in 1 X SDS-PAGE treatment solution, prior to electrophoresis, to produce a signal within a readable range. The assay was standardized by loading a series of treated solutions of known amounts of~~

~~pure FITC-Tf onto said electrophoresis gel. These consisted of 8 samples applied so that 2.4, 4.8, 9.7, 19, 39, 78, 156, and 313 ηg FITC-Tf protein were delivered per well, respectively. All samples were electrophoresed at 40 mA constant current until the dye front was 1 cm from the bottom of the gel. The gel was equilibrated in a transfer buffer of 48 mM Tris, 39 mM glycine. A 14 X 14 cm nitrocellulose membrane was equilibrated in transfer buffer and the gel and membrane assembled into a transfer apparatus and immersed in transfer buffer. Gel components were transferred to the membrane at a constant voltage of 40 V for 1.5h.~~

~~The membrane was blocked at 4°C overnight in a block solution consisting of Tris buffered saline (TBS: 25 mM Tris, 0.15 M NaCl, pH 7.8) containing 0.1% tween 20 and 5% w/v non-fat dry milk. The membrane was incubated with 1:1000 rabbit anti-FITC in block solution for 2h at 25°C, and washed three times (20 min each) with 50 ml TBS. The membrane was incubated with 1:2000 horse radish peroxidase conjugated goat anti-rabbit IgG in block solution for 2h at 25°C and washed again. The membrane was covered with an enhanced chemiluminescent substrate for horse radish peroxidase, was wrapped in plastic, and was loaded into an X-ray film cassette along with an 8 X 10 inch piece of chemiluminescent detecting film. The film was developed after 1 min exposure and an additional film was added which was developed after 20 min exposure. Bands produced on the film were quantitated by measurement of total optical density/mm^2, using a charged coupled device (CCD) camera equipped imager.~~

~~Figure 2A displays an image of the film from the transferrin binding method obtained after the 1 min exposure. Here, all lanes were loaded with lysates from equal quantities~~

~~of cells initially exposed to the concentration of FITC-TF listed above the blot. Signals from duplicate wells are shown. The figure shows that when increasing levels of FITC-Tf are initially present, that higher levels of FITC-Tf bind to a constant amount of cells, which is in keeping with normal binding behavior. In Figure 2B, the left half of the gel was loaded with cells initially treated as in Figure 2A, but also with 100 μg/ml of unconjugated Tf. The figure also displays markedly lower binding of FITC-Tf to the cells when an excess of Tf is initially present, indicating competition between FITC-Tf and Tf for cell binding, and therefore specific binding of FITC-Tf to the cells. The right half of Figure 2B shows results from the analysis of equal amounts of aliquots of the initial unbound samples from Figure 2A.~~

~~Figure 2C shows an image of the film obtained when standard solutions of FITC-Tf were electrophoresed, blotted, and analyzed for FITC content as indicated above. Here, the amounts of FITC-Tf loaded onto the electrophoresis gel (in ηg) are indicated on the top. Figure 2D displays the standard curve obtained when the density of the bands obtained from the image analysis of the film from Figure 2C were plotted against the amount of FITC-Tf present in each band. The equation shown on the curve was used to calculate the amount of FITC-Tf present in the bands from Figure 2A and 2B, thus enabling the determination of the weight of FITC-Tf bound per cell, and unbound per well, at each initial FITC-Tf concentration. This data was converted to molecules and moles, using a Tf molecular weight of 75,000. This data was then plotted as a conventional Scatchard analysis as shown in Figure 2F, to obtain Tf receptors per cell.~~

~~The binding of annexin V to cell surfaces has been recognized as an indicator of early~~

~~apoptosis (Zhang *et. al.*, 1997). With conventional procedures, cells are removed from plates, treated with FITC-annexin V, and analyzed by FACS. The removal of cells from tissue culture plates using conventional trypsin or EDTA reagents can in itself induce cell stress, apoptosis, and cellular annexin V binding (LeGall *et. al.*, 2000). Therefore, the conventional use of annexin V binding as a measure of apoptosis in adherent cells is problematic. In contrast, this new method would measure the binding of FITC-annexin V to adherent cultured cells *in situ* (Figures 3 and 4), where binding and washing occur first, before the cells are removed from plates for analysis. Therefore, the amount of FITC-annexin V detected would accurately represent that bound by cells in their natural culture environment. Thus, the method outlined in this new method circumvents conventional problems and provides for a more authentic measure of natural cellular annexin V binding.~~

~~A schematic of the strategy of the assay when used to detect apoptotic cells is shown in Figure 3. Cells in apoptosis (Figure 3A) or normal non-apoptotic cells (Figure 3B) are exposed to a solution of FITC-Annexin V. When washed (Figure 3C) and lysed, cells from Figure 3A produced a lysate containing FITC-Annexin V (Figure 3E), whereas cells from Figure 3B produce a lysate containing no FITC-Annexin V (Figure 3D, Figure 3F). In Figure 3G, both samples are loaded onto an electrophoresis gel, along with standards containing increasing levels of known amounts of FITC-Annexin V. When electrophoresed, the Annexin V and cell lysate proteins stack up and migrate according to their molecular weight (Figure 1-H). These are blotted onto a membrane as shown in Figure 3I, where their relative positions are maintained. The membrane is blocked and~~

~~treated with goat anti-FITC (Figure 3J). This specifically binds to the FITC-Annexin V only. The membrane is washed and treated with peroxidase-anti-goat IgG, which recognizes only the goat anti-FITC (Figure 3K). The membrane is washed again and treated with an ECL substrate for peroxidase (Figure 3L), where light produced per band correlates with membrane FITC-Annexin V quantity per band. The light produced is recorded on an X-ray film (Figure 3M), which is imaged so that each band is assessed for ODu/mm^2 (Figure 3N). With the cells from Figure 3A, plentiful FITC-Annexin V binds, this is then present on the blot, the initial antibody and therefore the second antibody bind, light is produced upon incubation with an HRP chemiluminescent substrate, and a band is seen on the film. The amount of FITC-Annexin V in this band can be estimated by comparing its signal to that of the standards. The amount of FITC-Annexin V bound per cell is calculated, from the cell density of the culture plate used in Figure 3A. With the cells from Figure 3B, no FITC-Annexin V binds, none is present on the blot, the initial antibody and therefore the second antibody do not bind, no light is produced upon incubation with an HRP chemiluminescent substrate, and no band is seen on the film.~~

~~The detailed methods for the lysis, electrophoresis, blotting, and ECL detection steps for annexin V, concanavalin A, Avidin, and insulin binding assays were the same as those outlined above in detail, for transferrin. The other specifics of these assays follow.~~

~~Figure 4 displays actual results obtained when this assay was used to measure the binding of FITC-Annexin V to rat MTLn3 mammary adenocarcinoma cells. The cells were grown to confluence in six well plates. Cells were induced to apoptose by treatment with 4 µg/ml Camptothecin (dissolved in DMSO). Controls received DMSO only. After~~

~~24 hours, wells were washed three times with and equilibrated in 1 ml binding buffer (25 mM HEPES, 0.15 M NaCl, 2.5 mM $CaCl_2$, pH 7.5). FITC-Annexin V was added to 50 η g/ml and the cells were incubated for 30 min at 25° C. Cells were then washed extensively with binding buffer, and lysed in 1 ml of RIPA lysing solution. The lysates were centrifuged at 5,000 X g for 5 min., the supernatants were assessed for total protein, and equal protein equivalents of the supernatants were treated (by the addition of one third volume 4X sample treatment solution and exposure to 95° C for 5 minutes) for, and separated by SDS-PAGE. Also run on the same gel were four pure FITC-Annexin V standards of 0.5, 1, 2, and 4 μg Annexin V protein per well. Separated proteins were blotted onto a nitrocellulose membrane which was blocked and then incubated with rabbit anti-FITC, and then with goat anti-rabbit IgG-HRP. HRP containing bands were then detected by ECL. Figure 4A shows a scan of the ECL detection film, with each lane marked at the top as to the sample applied. Figure 4B displays the standard curve obtained when the density of the bands obtained from the image analysis of the standards from Figure 4A were plotted against the amount of Annexin V present in each standard band. The equation shown on the curve was used to calculate the amount of Annexin V present in the bands from the cell lysates, thus enabling the determination of Annexin V bound per cell equivalent (or cell protein) for the various treatments (Figure 4C).~~

~~To further test and illustrate another embodiment of the assay, the ability of the method to detect the binding of Concanavalin A (Con A) to cells was examined. Figure 5 displays the results obtained when this assay was used to measure the binding of FITC-Con A to rat MTLn3 mammary adenocarcinoma cells. The cells were grown to~~

~~confluence in replicate, in six well plates. The cell number in three wells was determined by trypsinization of those cells followed by enumeration on a cell counter. The growth media of test wells was replaced with a binding buffer consisting of 25 mM HEPES buffered MEM containing 3 mg/ml liquid gelatin (as a carrier and blocking protein), at pH 7.5. The cultures were taken to 4° C and FITC-Con A was added to replicate wells so that the final concentrations of FITC-Con A were 0.1, 1.0, and 10.0 μg/ml. One well of each FITC-Con A concentration also received 200 μg/ml of native (un-conjugated Con A). The cells were incubated for 2h at 4° C, washed extensively with PBS, and lysed in 800 uL of RIPA lysing solution. The lysates were centrifuged at 5,000 X g for 5 min., and equal cell equivalents of the lysate supernatants were treated for (by the addition of one third volume 4X sample treatment solution and exposure to 95° C for 5 minutes) and separated by SDS-PAGE. Also run on the same gel were four pure FITC-Con A standards consisting of 1, 2, 4, and 8 ηg total FITC-Con A protein loaded per lane, respectively. Separated proteins were blotted onto a nitrocellulose membrane which was blocked and incubated with rabbit anti-FITC and then with goat anti-rabbit IgG-HRP. HRP-containing bands were detected by ECL onto an X-ray film. The film was imaged to obtain the optical density units/mm^2 of the bands. A scan of the film is shown in Figure 5A. Figure 5B displays the standard curve obtained when the density of the bands obtained from the image analysis of the standards from Figure 5A were plotted against the amount of FITC-Con A present in each standard band. The equation shown on the curve was used to calculate the amount of Con A present in the bands from the cell lysates, thus enabling the determination of Con A bound per cell equivalent for the~~

~~various treatments, as shown in Figure 5C. Cells which were initially treated with both FITC-Con-A and un-conjugated Con-A displayed markedly lower binding of FITC-Con A than cells which received FITC-Con-A only, indicating competition for binding between FITC-Con-A and unconjugated Con-A, further indicating specific cell binding by the FITC-Con-A.~~

~~The replacement of electrophoresis with dot-blot techniques is possible. This would require that the only immune-recognizable conjugated component present prior to dot-blotting would be the desired product and/or absolutely minimal interaction of either antibody with non-specific sample components~~

~~Figure 6 displays the results obtained when the samples from Figure 6 were analyzed by a dot blot procedure. For the standards, increasing volumes (2, 4, 8, and 16 µL) of a 100 ηg/ml FITC-Con-A solution were applied to a nitrocellulose membrane. For the lysates, 4 µL of lysates from cells treated with 0.1, 0.5, and 1.0 µg/ml FITC-Con-A (with or without an excess of native Con-A) were applied to the membrane. The membrane was blocked, incubated with rabbit anti-FITC, then with goat anti-rabbit IgG-HRP, and HRP-containing sites detected with ECL onto an X-ray film. The scans of the actual films from the standards are shown in Figure 6A, and that for the cell lysates in Figure 6B. The dots on the film were quantitated using an imager to obtain the optical density units/mm^2 of each dot. Figure 6C displays the standard curve obtained when the density of the bands obtained from the image analysis of the standards from Figure 6A were plotted against the amount of Con-A present in each standard dot. To maintain linearity, only the first three were used. The equation shown on the curve in Figure 6C was used to~~

~~calculate the amount of Con A present in the dots from the cell lysates in Figure 6B, thus enabling the determination of Con A bound per cell equivalent for the various treatments, as shown in Figure 6D. As with Figure 5, cells which were initially treated with both FITC-Con A and un-conjugated Con A displayed markedly lower binding of FITC-Con A than cells which received FITC-Con A only, indicating competition for binding between FITC-Con A and unconjugated Con A, further indicating specific cell binding by the FITC-Con A. This displays the usefulness of the technique in a dot blot procedure, where the SDS-PAGE and electroblotting steps are eliminated.~~

~~Another experiment designed to test and illustrate the use of the method, was one where the examination of the ability of the method to detect the binding of avidin to cells was conducted. Figures 7A - 7C display the results obtained when this assay was used to measure the binding of FITC-Avidin to rat MTLn3 mammary adenocarcinoma cells. The cells were grown to confluence in six well plates. The cell number in three wells was determined by trypsinization of those cells followed by enumeration on a cell counter. The growth media was replaced with a binding buffer consisting of 25 mM HEPES buffered MEM containing 3 mg/ml liquid gelatin (as a carrier and blocking protein), at pH 7.5. The cultures were taken to 4° C and FITC-Avidin was added to replicate wells so that the final concentrations of FITC-Avidin were 0.1, 0.2, and 0.4 µg/ml. The cells were incubated for 2h at 4° C, washed extensively with PBS, and lysed in 1 ml of RIPA lysing solution/well. The lysates were centrifuged at 5,000 X g for 5 min., and the supernatants representing equal cell equivalents were treated (by the addition of one third volume 4X sample treatment solution and exposure to 95° C for 5 minutes) and separated~~

~~by SDS-PAGE. Also run on the same gel were four pure FITC-Avidin standards of 100, 200, 400, and 800 pg total FITC-Avidin protein loaded per lane, respectively. Separated proteins were blotted onto a nitrocellulose membrane which was blocked and incubated with rabbit anti-FITC and then with goat anti-rabbit IgG-HRP. HRP containing bands were detected by ECL onto an X-ray film. The film was imaged to obtain the optical density/mm^2 (ODu/mm^2) of the bands. A scan of the film is shown in Figure 7A. Figure 7B displays the standard curve obtained when the density of the bands obtained from the image analysis of the standards from Figure 7A were plotted against the amount of FITC-Avidin present in each standard band. The equation shown on the curve was used to calculate the amount of FITC-Avidin present in the bands from the cell lysates, thus enabling the determination of FITC-Avidin bound per cell equivalent for the various treatments, as shown in Figure 7C.~~

~~To further test the versatility of the method, its ability to detect the cellular binding of the low molecular weight protein insulin was examined. Figure 8 displays the results obtained when this assay was used to measure the binding of FITC-Insulin to human K562 erythroleukemia cells. Logarithmically growing cells in suspension culture were collected by centrifugation and washed twice by suspension in and centrifugation from a binding buffer consisting of alpha-MEM containing 5 mg/ml BSA and 25 mM HEPES (pH 7.5). Cells were adjusted to a density of 2 X 10^6/ml (in binding buffer), and were equilibrated to 4° C. To 1 ml of cell suspension was added 20 µl of 1 mg/ml FITC-Insulin (in binding buffer; final concentration in cell suspension = 20 µg/ml). An additional tube also received non-conjugated Insulin at a level of 200 µg/ml. Cell~~

~~suspensions were incubated for 2h at 4° C while rotating slowly, and the cells were collected and washed three times by suspension in and centrifugation from binding buffer. The initial supernatants were kept as the unbound samples. Cell pellets were lysed in 0.4 ml/tube of Schagger-Von Jagow (SVJ) electrophoresis system treatment solution (50 mM Tris-HCl, 2% w/v SDS , 1% v/v beta-mercaptoethanol, 5% v/v glycerol, 0.1% w/v bromophenol blue, pH 6.8), and treated at 95° C for 5 min. Unbound samples were likewise treated by the addition of one third volume of a 4X concentrate of the sample treatment solution, and exposure to 95° C for 5 min. Lysates (100 µl each) and aliquots of the treated unbound samples were separated by SDS-PAGE run according to Schagger-Von Jagow. These gels consisted of a 12 X 10 cm separating gel containing 1M Tris, 0.1% w/v SDS, 12% w/v acrylamide, pH 8.45; and a 12 X 2 cm stacking gel containing 0.75M Tris, 0.1% w/v SDS, 4% w/v acrylamide, pH 8.45. Gels were run in an electrode buffer of 0.1M Tris, 0.1M Tricine, 0.1 % SDS. Also run on the same gel were three treated pure FITC-Insulin standards of 1, 2, and 4 ηg total FITC-Insulin protein loaded per lane, respectively. Gel components were transferred to a nitrocellulose membrane which was blocked and incubated with rabbit anti-FITC and then with goat anti-rabbit IgG-HRP. HRP containing bands were detected by ECL onto an X-ray film. In the Figure 8, a scan of the X-ray film is shown. The lanes of the membrane are shown at the top, and the samples that those lanes received are shown at the bottom. Increasing signal is returned for increasing loads of FITC-insulin in the standards (Figure 8, lanes 1-3). The FITC-insulin bound by the cells is easily observed (Figure 8, lane 4), and this is reduced significantly when excess un-conjugated insulin was present (Figure 8, lane 5).~~

~~This procedure consistently displays higher molecular weight forms of insulin formed after application to cells, perhaps due to the presence of insulin binding proteins (Figure 8, lanes 4-7).~~

~~General applications: The assay strategy can apply to any ligand conjugated with a compound which can be specifically recognized by an antibody. In particular, anti-digoxygenin, anti-rhodamine and anti-biotin antibodies exist which would recognize ligands conjugated with those compounds. The material to which the ligand binds to can be other than cells. Any particles or other insoluble material can serve as the binding surface. Centrifugation and re-suspension of suspended particulate binding substrates would serve as a method for washing those of unbound ligand. The current method requires that the bound ligand be removed from the binding surface so that it can be separated by electrophoresis. It must also bind to a conventional transfer membrane for detection with the antibody. Other specific applications accomplished to date include the study of the binding of FITC-conjugated concanavalin A to cells, the study of the binding of Avidin to cells, the study of the binding of Annexin V to cells, and the study of the binding of insulin to cells. With the Annexin V protein, this assay could be utilized to assess cellular apoptosis without the need for a FACS analyzer.~~

~~In another embodiment, the assay could be used to verify the hybridization of a known biotin-labeled DNA to a surface. After binding, the bound labeled DNA is released through heat de-naturation, is separated by agarose electrophoresis, electro-blotted to nytran, and is detected by incubation with species x anti-biotin followed by incubation with anti-species x IgG-HRP and ECL. The final result yields a major band at~~

~~the expected bp size of the labeled DNA. As with the above stated protein procedures, the proper molecular weight of the desired product is verified by comparison to standards of the labeled DNA run on the same electrophoresis gel.~~

~~Conclusion: this new method is a procedure for measuring the binding of an entity (ligand) to a surface by using a hapten conjugated version of the ligand (hapten-ligand), where the hapten is recognizable by an antibody. An excess of the hapten-ligand is presented to the binding surface and excess (unbound) hapten-ligand is washed off. Bound hapten-ligand is then solubilized (removed) and applied to a membrane support or separated by electrophoresis and applied to a membrane support. Known quantities of the hapten-ligand are also applied to a membrane support or separated by electrophoresis and applied to a membrane support. The membrane-bound hapten-ligand is detected by application of an enzyme-conjugated antibody to the hapten; or by application of an antibody to the hapten followed by application of an enzyme-conjugated antibody to the anti-hapten antibody. The resultant membrane-associated enzyme is detected and quantitated by the application of a color or light-producing substrate which reacts with the enzyme. Results obtained from the standards are used to construct a standard curve which is then used to calculate the amount of hapten-ligand in the membrane areas corresponding to the unknowns. Thus, the amount of hapten-ligand originally bound to the surface can be determined. This assay method has the advantages of providing verification of the molecular weight of the binding substance (ligand) via the electrophoresis step. It eliminates the need for radioactive materials. The procedure provides for high sensitivity detection as the dual antibody incubation steps amplify the~~

~~signal significantly. The procedure allows for easy standardization as different user-definable levels of a standard solution of the Hapten-ligand can be simultaneously applied to the electrophoresis gel or to the dot-blot or slot-blot membrane~~